Recruited Inflammatory Cells Mediate Endotoxin-induced Lung Maturation in Preterm Fetal Lambs

Suhas G. Kallapur, Timothy J. M. Moss, Machiko Ikegami, Richard L. Jasman, John P. Newnham, and Alan H. Jobe

Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio; School of Women's and Infants' Health, University of Western Australia, Perth, Australia; and ICOS Corporation, Bothell, Washington

Rationale: **Chorioamnionitis is paradoxically associated with a decreased incidence of respiratory distress syndrome in preterm infants. In preterm lambs, intraamniotic endotoxin and interleukin 1 (IL-1) induce lung inflammation followed by lung maturation.**

Objective: **To test if inflammatory cells are required to mediate induced lung maturation.**

Methods: **Lung inflammation was induced by intraamniotic injection of endotoxin or IL-1. Inflammatory cell recruitment to the lung was inhibited by an anti-CD18 blocking antibody given intramuscularly to the fetus. Preterm lambs were delivered at 124-d gestation (term = 150 d) 2 or 7 d after exposure to endotoxin/IL-1 or endotoxin/IL-1 + anti-CD18 antibody.**

Measurements: **Lung inflammation was measured by bronchoalveolar lavage fluid cell count, inflammatory scoring of lung parenchyma, and expression of proinflammatory cytokines and inducible nitric oxide synthase. Lung maturation was quantitated by surfactant protein mRNA expression, saturated phosphatidylcholine pool size, and pressure–volume curves.**

Main Results: **Inhibition of CD18 significantly reduced endotoxin-induced but not IL-1–induced fetal lung inflammatory cell recruitment and activation as well as expression of proinflammatory cytokines. Compared with control lungs, both endotoxin and IL-1 induced lung maturation. Anti-CD18 antibody administration inhibited only endotoxin-induced but not IL-1–induced increases in surfactant protein mRNA and surfactant saturated phosphatidylcholine. Exposure to anti-CD18 antibody moderated endotoxin-induced increases in lung volumes but had no effect on IL-1–induced increases in lung volumes.**

Conclusions: **(*1*) Endotoxin- but not IL-1–induced inflammatory cell recruitment in the preterm fetal lamb lung is CD18 dependent; (*2*) recruited inflammatory cells mediate some aspects of fetal lung maturation.**

Keywords: bronchopulmonary dysplasia; CD18; chorioamnionitis; respiratory distress syndrome; surfactant

Chorioamnionitis or infection/inflammation of the fetal membranes and amniotic fluid is a pregnancy complication associated with up to 70% of all preterm deliveries at less than 30-wk gestation (1). Clinical and epidemiologic studies suggest that chorioamnionitis can decrease the risk for respiratory distress syndrome due to surfactant deficiency in preterm infants (2, 3). Pulmonary surfactant, a product of the alveolar type II cells, is the essential substance that lowers surface tension at the air–liquid interface in the lung. Surfactant is composed of about 90% lipids (primarily saturated phosphatidylcholines [Sat PCs]) and 10% surfactant-associated proteins A, B, C, and D (4).

In preterm fetal lamb models, intraamniotic (IA) endotoxin caused chorioamnionitis, improved lung compliance, increased surfactant lipids, and increased surfactant protein mRNA and protein levels (5, 6). The maturational effects occurred 7 d after IA endotoxin, whereas maximum inflammatory cell influx in the fetal lung occurred 2 d after IA endotoxin (7). IA injections with interleukin 1α (IL-1α) or live *Ureaplasma parvum* also caused chorioamnionitis, inflammatory cell influx into the fetal lamb lung followed by increased surfactant pool sizes (8, 9). These experiments suggest that chorioamnionitis-induced inflammatory cell influx in the lung is associated with increased surfactant and fetal lung maturation. However, it is not known if inflammatory cells are required for the induced lung maturation.

The members of the β_2 (CD18) subfamily of integrins expressed on leukocytes are as follows: lymphocyte function–associated antigen-1 (CD11a/CD18), Mac-1 (CD11b/CD18), p150,95 (CD11c/CD18), and α4/CD18 (10, 11). The leukocyte β_2-integrins bind to intercellular adhesion molecule (ICAM)-1, an adhesion molecule in the immunoglobulin supergene family that is expressed on the vascular endothelium (12). With inflammation, endothelial ICAM-1 increases and, via its interactions with leukocyte β_2-integrins, causes recruitment of leukocytes into the tissues (13, 14). Although there are a number of different mechanisms regulating pulmonary influx of leukocytes, studies in mice and humans with leukocyte adhesion defects suggest that leukocyte CD18 plays an important role in the influx of leukocytes into lungs exposed to *Escherichia coli* (14, 15). The mechanisms of influx of leukocytes into the fetal lungs are unknown.

We tested the hypothesis that a blocking anti-CD18 antibody would decrease the fetal lung leukocyte influx in response to *E. coli* endotoxin and IL-1 and decrease the lung maturation induced by these agents. Preterm fetal lambs were exposed to IA *E. coli* endotoxin with or without a blocking anti-CD18 antibody. A parallel experiment was performed with IL-1α as the inflammatory stimulus. The lung inflammation was assessed at 2 d at the peak of the inflammatory response. Lung function and surfactant were measured 7 d after IA endotoxin or IL-1α.

This article has been previously published in abstract form (16, 17).

METHODS

Additional details on methods are provided in an online supplement.

Animals and Treatments

Date-mated Merino ewes with singleton gestations were used after approval by the appropriate animal care and use committees of the

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Correspondence and requests for reprints should be addressed to Suhas G. Kallapur, M.D., Cincinnati Children's Hospital Medical Center, University of Cincinnati, Division of Pulmonary Biology, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. E-mail: suhas.kallapur@cchmc.org

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Western Australian Department of Agriculture and Cincinnati Children's Hospital Medical Center. All treatments were randomly assigned. One 50-mg dose of anti-CD18 antibody (23I11B, 7.66 mg/ml, IgG2a isotype; ICOS Corp., Bothell, WA) or saline was given by fetal intramuscular injection at 122-d gestation to the endotoxin 2-d group and the IL-1α 2-d group, and at 117-d gestation to the endotoxin 7-d group (*see* Table 1 for animal groups). For the 7-d IL-1α group and anti-CD18 antibody–alone group, 50 mg anti-CD18 antibody was given at 117 d and repeated at 121- and 123-d gestation since antibody levels decreased 7 d after one dose in the endotoxin group. IA injections with 10 mg *E. coli* endotoxin (055:B5; Sigma, St. Louis, MO) or sheep recombinant IL-1α (100 μg; Protein Express, Cincinnati, OH) or saline were given 3 h after the first dose of anti-CD18 antibody or saline fetal intramuscular injections. Fetal IA and intramuscular injections were given using ultrasound guidance (5).

Delivery, Bronchoalveolar Lavage Fluid Collection, and Pressure–Volume Curves

Fetal lambs receiving injections at 122 d were delivered 2 d later and those receiving injections at 117 d were delivered 7 d later at 124 d (term = 150 d). The lambs were delivered by hysterotomy; deflation limb pressure–volume curves were measured. Bronchoalveolar lavage fluid (BALF) and lung pieces were collected for cell count, surfactant lipid, RNA, protein, and morphologic studies as described (18).

Sat PC Measurement

Aliquots from the pooled lavage samples or homogenized lung pieces were extracted with chloroform and methanol (2:1) (19). The lipid extracts were treated with osmium tetroxide, and the major surfactant lipid, Sat PC, was recovered after alumina column chromatography and quantified by phosphorus assay (20, 21).

RNA Extraction and RNA Quantitation

Total RNA was isolated using a modified Chomzynski method, and 10 μg of total RNA was used for IL-1β, IL-6, and IL-8 quantitation using RNase protection analysis (7). Surfactant protein A (SP-A), SP-B, and SP-C mRNA was quantitated using 3 μg total RNA by the S1 nuclease protection assay (6).

ELISA

IL-8 protein was quantitated in the BALF by an ELISA assay (22). Plasma anti-CD18 antibody levels were measured using antimouse IgG specific antibodies (Sigma), with no sheep cross-reactivity in an ELISA assay (23).

Flow Cytometry

Peripheral blood leukocytes obtained by hypotonic lysis of whole blood or alveolar macrophages in BALF from ewes were used for flow cytometric evaluation of anti-CD18 antibody reactivity and dose response.

Inducible Nitric Oxide Synthase Immunohistochemistry and Scoring of Lung Inflammation

Lung inflammation in parenchyma and lumen was scored in a blind manner. Inflammatory cells were identified by morphologic characteristics or inducible nitric oxide synthase (iNOS) immunostaining using monoclonal anti-iNOS antibody (1:250; Transduction Labs, Lexington, KY) using antigen retrieval as described (24).

Statistics

Data were expressed as dot plots with median values or mean ± SEM. Comparisons between different groups were made with two-tailed unpaired *t* tests, two-tailed Mann-Whitney nonparametric tests, or two-way Kruskal-Wallis nonparametric analysis of variance as appropriate. Significance was accepted at $p < 0.05$.

RESULTS

Cross-Reactivity of Anti-CD18 Antibody 23I11B

The suitability of the 23I11B antibody for *in vivo* studies in lambs and target plasma concentrations were established *in vitro* using sheep alveolar macrophages and peripheral blood leukocytes. Flow cytometry was used to demonstrate cross-reactivity of the anti-CD18 antibody (Figure 1A) and expression of CD18 on peripheral blood neutrophils, monocytes, and lymphocytes (not shown). A dose–response evaluation using alveolar macrophages showed that 70% of the maximum receptor saturation (mean fluorescent intensity of staining) was observed at 20 μg/ml and 80% at 100 μg/ml. The receptor saturation decreased below 4 μg/ml (Figure 1B). Therefore, a plasma antibody concentration between 20 and 100 μg/ml was targeted. With a single dose of 50 mg given to the fetal sheep (weighing ~ 3 kg), the mean plasma concentrations of anti-CD 18 antibody (mouse IgG) 2 and 7 d after injection were 49 and 5.3 μg/ml, respectively (Figure 1C).

Physiologic Variables at Birth

The control lambs given saline, lambs given anti-CD18 antibody alone, and all other treated groups had similar bodyweights, lung/bodyweights, and cord blood gases (Table 1). There were no fetal deaths.

Peripheral Blood Leukocytes and Platelets

Compared with control lungs, IA endotoxin and IL-1α increased total white blood cells, neutrophils, lymphocytes, monocytes, and platelets at 7 d (Table 2). Compared with the corresponding endotoxin or IL-1α groups, administration of anti-CD18 antibody did not change the total or differential white blood cell or platelet counts.

TABLE 1. PHYSIOLOGIC VARIABLES OF PRETERM LAMBS AT BIRTH

Group	n	Bodyweight (*kg*)	Lung/Bodyweight	Cord Blood pH	Cord Blood P_{CO_2} (*mm Hg*)
Control (saline)	6	2.98 ± 0.10	0.039 ± 0.002	7.38 ± 0.01	55.5 ± 3.1
Anti-CD18 antibody alone	7	2.35 ± 0.09	0.032 ± 0.001	7.35 ± 0.01	58.1 ± 2.2
Endotoxin 2 d	4	2.98 ± 0.10	0.045 ± 0.002	7.32 ± 0.03	65.8 ± 5.6
Endotoxin 2 d + anti-CD18	6	2.81 ± 0.10	0.039 ± 0.002	7.37 ± 0.01	58.4 ± 1.5
Endotoxin 7 d	4	2.58 ± 0.1	0.045 ± 0.002	7.38 ± 0.01	55.7 ± 1.4
Endotoxin 7 d + anti-CD18	4	3.28 ± 0.03	0.041 ± 0.003	7.39 ± 0.01	50.1 ± 1.1
IL-1 2 d	4	2.67 ± 0.1	0.032 ± 0.002	7.37 ± 0.01	58.7 ± 1.2
IL-1 2 d + anti-CD18	6	2.34 ± 0.3	0.034 ± 0.003	7.32 ± 0.01	65.9 ± 3.2
IL-1 7 d	7	2.54 ± 0.05	0.033 ± 0.002	7.36 ± 0.01	57.9 ± 1.5
IL-1 7 d + anti-CD18	7	2.40 ± 0.10	0.036 ± 0.002	7.36 ± 0.01	55.7 ± 1.5

Definition of abbreviation: IL = interleukin.
Lambs in all the groups were delivered at 124- ± 1-d gestational age.

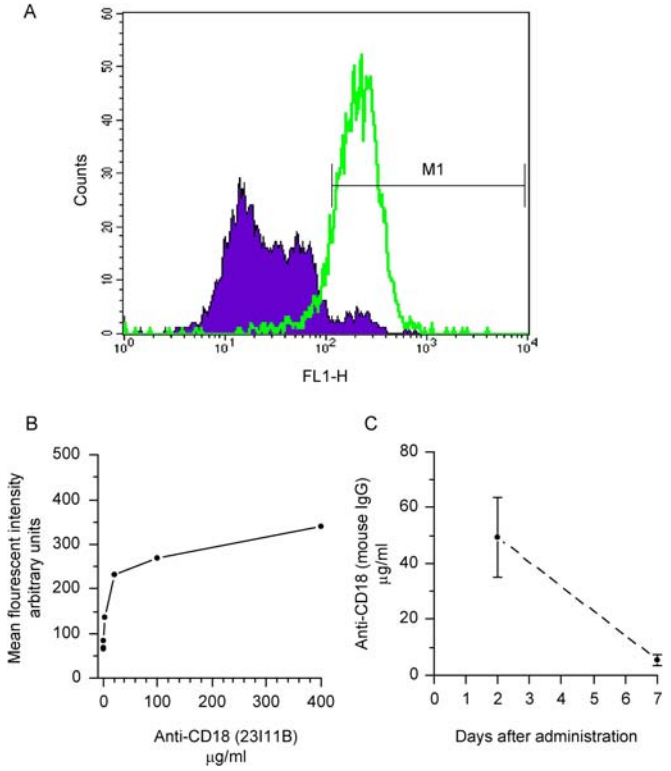

Figure 1. The anti-CD18 antibody 23I11B binds sheep CD18. (*A*) Fluorescence-activated cell sorter analysis was performed on the bronchoalveolar lavage fluid (BALF) adult sheep alveolar macrophages using anti-CD18 (23I11B) antibody. Compared with control lungs (*shaded area*), anti-CD18 antibody caused a shift in fluorescein isothiocyanate staining (*open area*) shown with a log scale on the *x axis*. (*B*) A dose–response curve of one representative experiment performed in duplicate shows mean fluorescent intensity of staining versus anti-CD18 antibody concentration. The mean fluorescent intensity of staining plateau was observed between 20 and 100 μg/ml of the 23I11B antibody concentration. (*C*) Plasma concentration of anti-CD18 antibody in preterm lambs 2 and 7 d after one 50-mg fetal intramuscular dose (4–6 animals/group).

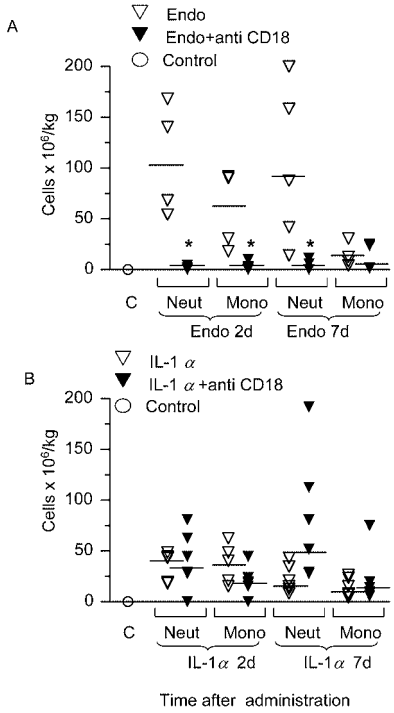

Figure 2. The anti-CD18 antibody decreases intraamniotic (IA) endotoxin (Endo) but not interleukin (IL)-1α–induced neutrophil and monocyte influx in the lung. (*A*) IA Endo and (*B*) IA IL-1α induced inflammatory cell influx in the BALF of preterm fetal lambs 2 and 7 d after exposures. Cell counts were expressed per kilogram of bodyweight and are shown as *dot plots* with median values. Neutrophils (Neut) and monocytes (Mono) were not detected in control BALF. Endo- but not IL-1α–induced neutrophil and monocyte influx was blocked by anti-CD18 antibody pretreatment (C = control, 4–6 animals/group; *p < 0.05 vs. Endo).

Anti-CD18 Antibody Inhibits Endotoxin-induced Inflammation in the Fetal Lung

Fetal lung inflammation was evaluated by recruitment of leukocytes in the lung, BALF, expression of proinflammatory cytokines, and iNOS. Consistent with our previous results (7) and relative to control lungs, BALF neutrophils and monocytes increased 2 and 7 d after IA endotoxin (Figure 2A). Pretreatment with anti-CD18 antibody almost completely inhibited endotoxin-induced recruitment of neutrophils into BALF at 2 and 7 d and monocytes at 2 d.

Consistent with the BALF data, very few neutrophils and monocytes were detected in the lung parenchyma of the control lungs (Figures 3A and 3B). Endotoxin increased inflammatory cells in the fetal lungs. Pretreatment with the anti-CD18 antibody decreased lung inflammatory cells 2 and 7 d after IA endotoxin (Figure 3A). Activation of inflammatory cells was assessed by iNOS immunostaining. The iNOS expression in the inflammatory cells was detected at 2 d and had decreased 7 d after IA endotoxin (*compare* Figures 3C and 3E). Pretreatment with the anti-CD18 antibody decreased IA endotoxin–induced iNOS expression in the inflammatory cells 2 and 7 d after IA endotoxin (*compare* Figures 3C and 3D–3F).

Previous experiments showed maximum proinflammatory cytokine mRNA expression at 2 d with a return to control levels 4 to 7 d after IA endotoxin (7). In this experiment, the mRNAs for proinflammatory cytokines IL-1β, IL-8, and IL-6 also were induced in the fetal lung 2 d after IA endotoxin (Figures 4A–4C). Consistent with fewer lung inflammatory cells, pretreatment with anti-CD18 antibody decreased proinflammatory cytokine IL-8 and IL-6 mRNA (Figures 4B–4C). Similarly, pretreatment with anti-CD18 antibody decreased the IA endotoxin–induced increase

TABLE 2. TOTAL AND DIFFERENTIAL WHITE BLOOD CELL COUNTS IN THE PERIPHERAL BLOOD

Group	Total WBC (10^9/L)	Neutrophils (10^9/L)	Lymphocyte (10^9/L)	Monocyte (10^9/L)	Platelets (10^9/L)
Control (saline)	3.8 ± 0.4	0.6 ± 0.1	1.5 ± 0.2	0.2 ± 0.02	536 ± 28
Anti-CD18 antibody alone	2.8 ± 0.4	0.2 ± 0.1*	2.0 ± 0.3	0.03 ± 0.0*	546 ± 53
Endotoxin 7 d	6.2 ± 0.9*	2.9 ± 0.8*	2.46 ± 0.38*	0.3 ± 0.05*	752 ± 91*
Endotoxin 7 d + anti-CD18	5.6 ± 1.4	2.1 ± 1.1	2.46 ± 0.42	0.16 ± 0.07	498 ± 154
IL-1 7 d	6.7 ± 0.8*	3.8 ± 1.1*	4.7 ± 0.6*	0.07 ± 0.0*	881 ± 57*
IL-1 7 d + anti-CD18	6.1 ± 1.2*	2.5 ± 0.6*	4.2 ± 0.8*	0.06 ± 0.01*	838 ± 80*

Definition of abbreviations: IL = interleukin; WBC = white blood cell.
* $p < 0.05$ versus control.

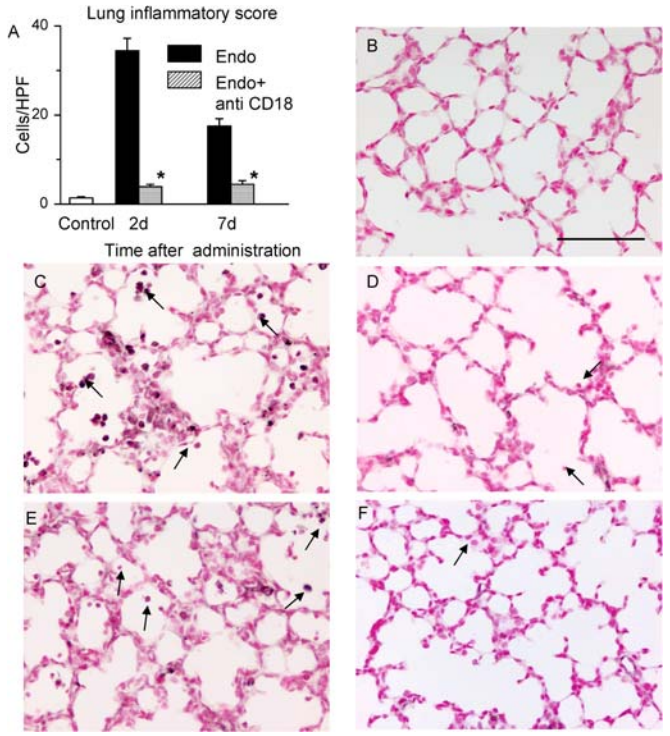

Figure 3. Anti-CD18 antibody decreases IA Endo–induced inflammation in the lung. (*A*) Inflammatory score in airspace + parenchyma 2 and 7 d after IA Endo and anti-CD18 antibody treatment. Representative lung sections from (*B*) control, (*C*) IA Endo 2 d, (*D*) IA Endo + anti-CD18 antibody 2 d, (*E*) IA Endo 7 d, and (*F*) IA Endo + anti-CD18 antibody 7 d were used for inducible nitric oxide synthase (iNOS) immunostaining. The *arrows* point to neutrophils/monocytes. iNOS immunostaining was the strongest in the IA Endo 2-d group and decreased in the IA Endo 7-d group. In the few neutrophils/monocytes in the anti-CD18 antibody group, iNOS immunoreactivity was barely detectable. Endo-induced iNOS expression was blocked by anti-CD18 antibody pretreatment. Three animals/group and 10 nonoverlapping high-power fields/animal were blindly scored; *bar* represents 50 μm; *$p < 0.05$ vs. Endo.

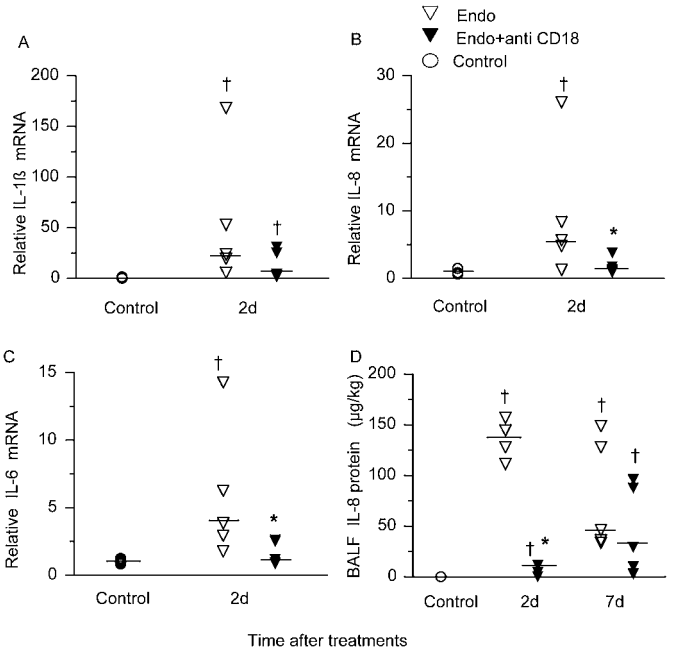

Figure 4. Anti-CD18 antibody decreases IA Endo–induced cytokine expression in the lung. Endo induced expression of cytokine mRNA. (*A*) IL-1β, (*B*) IL-8, (*C*) IL-6 mRNA in total lung, and (*D*) IL-8 protein in BALF were measured. The mRNAs were quantified by RNase protection assays using lung from preterm lambs 2 d after IA Endo. The mRNA values were normalized to L32 (ribosomal protein mRNA). The mean mRNA signal in control animals was given a value of 1, and levels at each time point were expressed as mRNA values relative to controls. IL-8 protein in the BALF was quantitated by ELISA and expressed per kilogram of bodyweight. Data are shown as a *dot plot* with median values. Endo-induced cytokine expression was blocked by the anti-CD18 antibody pretreatment (4–6 animals/group, *$p < 0.05$ vs. Endo, †$p < 0.05$ vs. controls).

in the BALF IL-8 protein level 2 d after exposure (Figure 4D). The inhibition of BALF IL-8 protein was not sustained at 7 d.

Effects of Anti-CD18 Antibody on Endotoxin-induced Lung Maturation

Lung maturation was assessed by quantitating SP mRNA, Sat PC, and lung volume. Compared with control lungs, IA endotoxin increased the mRNAs for SP-A 10-fold, SP-B 3.5-fold, and SP-C 1.5-fold in the fetal lung at 2 d (Figure 5). Pretreatment with anti-CD18 antibody did not significantly decrease the SP mRNA induction at 2 d. However, compared with the IA endotoxin group, the lambs in the IA endotoxin + anti-CD18 group had significantly less induction of SP-A, SP-B, and SP-C mRNA in the fetal lung at 7 d. The reductions in the anti-CD18 antibody–exposed group as a fraction of the endotoxin group were as follows: SP-A mRNA (47%), SP-B (63%), and SP-C (65%).

Compared with control lungs, the Sat PC pool size was increased in the BALF and in the lung tissue 7 d after IA endotoxin (Figures 6A and 6B). Pretreatment with anti-CD18 antibody decreased the IA endotoxin–mediated induction of Sat PC both in the BALF and the lung. Compared with control lungs, IA endotoxin increased lung gas volumes (Figure 6C). The mean pressure–volume curve for the IA endotoxin group + anti-CD18 antibody was intermediate between the control lungs and IA endotoxin group.

Anti-CD18 Antibody Does Not Inhibit IL-1α–induced Fetal Lung Inflammation or Lung Maturation

IA endotoxin induces IL-1β expression in the fetal lung and IA IL-1α causes lung inflammation and maturation (7, 8). Therefore, we evaluated if the CD18–ICAM-1 interaction also mediates IL-1–induced lung inflammation and maturation. In contrast to endotoxin effects, anti-CD18 antibody did not decrease IL-1–mediated neutrophil and monocyte influx in the BALF of preterm lambs 2 or 7 d after exposure (*compare* Figure 2B with Figure 2A).

Consistent with its inability to decrease IL-1–induced fetal lung inflammation, anti-CD18 antibody did not inhibit the IL-1–induced 21-fold increase in BALF Sat PC (Figure 7A) or SP mRNAs (not shown). Similarly, anti-CD18 antibody also did not decrease the IL-1–induced 3.2-fold increase in lung volume at 40 cm H_2O pressure (Figure 7B).

DISCUSSION

Antenatal exposure of preterm infants to infection and inflammation can result in adverse fetal consequences, such as lung injury (bronchopulmonary dysplasia) and brain injury (periventricular leukomalacia) (25, 26). However, paradoxically, exposure to

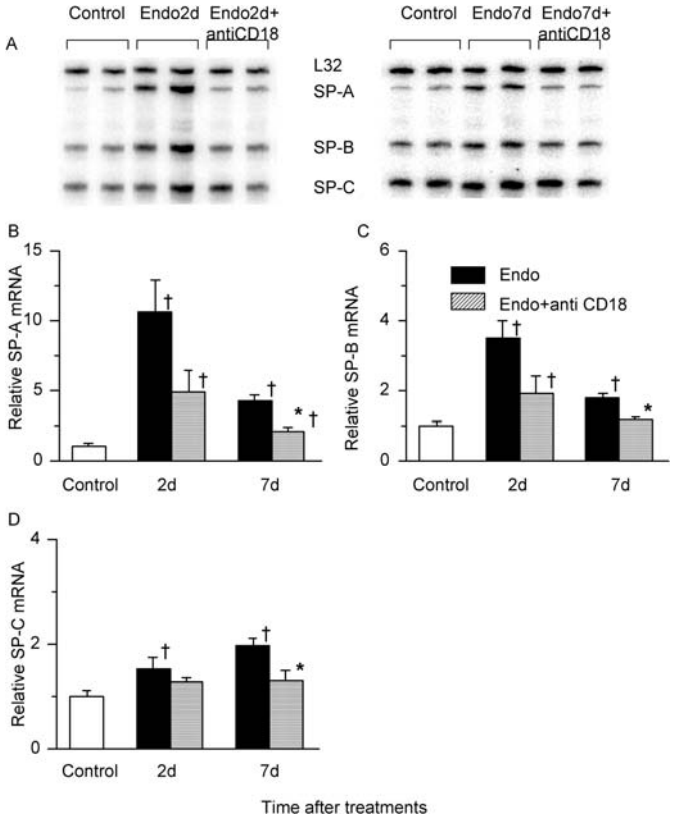

Figure 5. Effects of anti-CD18 antibody on surfactant protein mRNAs. (*A*) Representative S1 nuclease protection assay of surfactant protein A (SP-A), SP-B, and SP-C mRNA in preterm lamb lungs. Quantitation of SP mRNAs: (*B*) SP-A, (*C*) SP-B, and (*D*) SP-C mRNA 2 and 7 d after IA Endo and anti-CD18 antibody treatment. Total lung RNA (3 μg) from lung was used for S1 nuclease protection assay, and the mRNA values were normalized to L32 (ribosomal protein mRNA). The mean mRNA signal in control animals was given a value of 1, and levels at each time point were expressed as mRNA values relative to control lungs. Exposure to the anti-CD18 antibody decreased Endo-induced increases in SP mRNAs at 7 but not at 2 d (4–6 animals/group, *$p < 0.05$ vs. Endo, †$p < 0.05$ vs. controls).

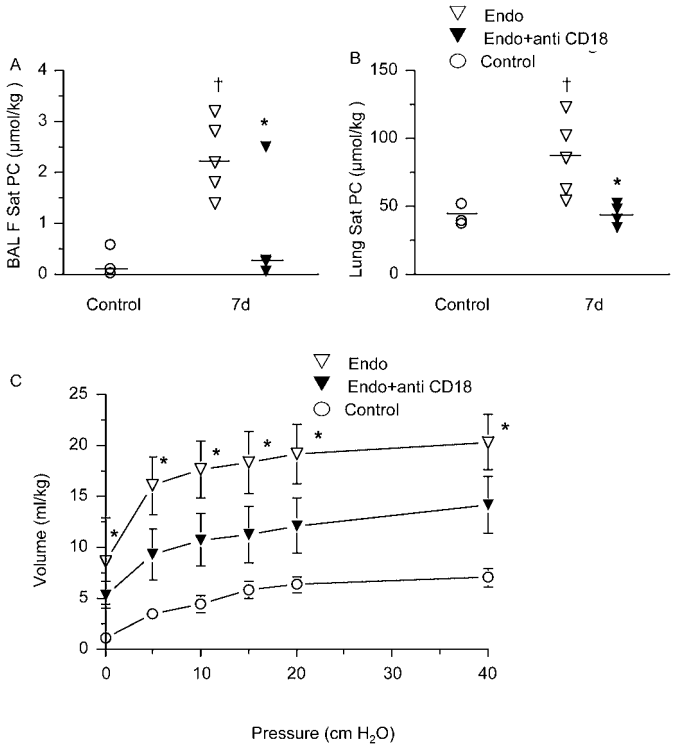

Figure 6. Anti-CD18 antibody decreases IA Endo–induced increases in surfactant lipids in the lung. (*A*) Saturated phosphatidylcholine (Sat PC) concentration in BALF and (*B*) lung homogenate were expressed as μmol/kg bodyweight. (*C*) The pressure–volume curve was measured by lung volumes expressed as ml/kg bodyweight versus pressure in cm H_2O. IA Endo significantly increased lung volumes compared with control preterm lambs. Data for Sat PC pool size are shown as *dot plots* with median values, and lung volumes are shown as mean ± SEM values. Anti-CD18 antibody exposure decreased BALF and total lung Sat PC 7 d after IA Endo. The lung volumes after anti-CD18 antibody pretreatment were intermediate between IA Endo and controls (4–6 animals/group, *$p < 0.05$ vs. Endo, †$p < 0.05$ vs. controls).

antenatal inflammation also results in clinical lung maturation (increased airway surfactant pools and improved lung mechanics) (7). A widely used clinical strategy to induce fetal lung maturation is the use of maternal glucocorticoids. Compared with maternal glucocorticoids, fetal exposure to endotoxin causes a more robust and longer lasting induction of surfactant synthesis and improved lung mechanics (27). Inflammation in the fetal lamb lung caused by IA endotoxin, IL-1α, or *U. parvum* also induce lung maturation without an increase in fetal plasma cortisol (5, 8, 9). Clinically, multiple organisms are associated with chorioamnionitis. Thus, the pathways and mechanisms involved in inflammation-induced clinical lung maturation are of considerable clinical interest.

We have used endotoxin as an inflammatory agonist to characterize the kinetics of induced fetal lung inflammation and maturation (6, 7). After IA endotoxin exposure, inflammatory cell influx and lung proinflammatory cytokine expression are maximal at 2 d, with decreases in proinflammatory cytokine mRNA expression to control levels but persistence of BAL inflammatory cells at 7 d. In the present study, anti-CD18 antibody almost completely blocked neutrophil and monocyte influx and activation in the lung 2 d after endotoxin exposure. The lung inflammation was partially blocked by anti-CD18 antibody at 7 d because neutrophil counts were decreased but monocyte counts and IL-8 protein in the BALF were similar to control lungs. The half-life of endotoxin in the amniotic fluid is about 30 h (28), and a dose of 1 mg IA endotoxin can induce lung maturation (29). We interpret the incomplete blockade of lung inflammation by the anti-CD18 antibody at 7 d to be due to falling antibody levels with continued IA endotoxin exposure.

The SP-A, SP-B, and SP-C mRNAs are maximally induced at 2 d after endotoxin exposure (6). The alveolar and lung Sat PC pool sizes increase at 7 d but not at 2 d after endotoxin exposure. The increased surfactant pools correlate with improved lung compliance in prematurely delivered lambs, 7 d after IA endotoxin (clinical lung maturation). The endotoxin-induced increase in BALF and lung Sat PC pool size was almost completely blocked by anti-CD18 antibody. The induction of SP mRNA expression relative to the endotoxin-exposed group was partially blocked at 7 d but not at 2 d. This difference in effects at 2 versus 7 d is likely explained by the larger variation in the induction of SP mRNA within the antibody-exposed group at 2 d compared with the 7-d group. The small increase in surfactant lipid pool size in the antibody- and endotoxin-treated group relative to control lungs probably resulted in lung compliance

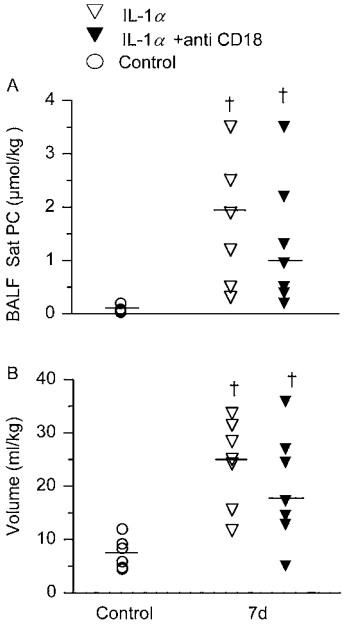

Figure 7. Anti-CD18 antibody did not change the IL-1α–induced increase in surfactant lipids in the lung. (*A*) Sat PC concentration in BALF and (*B*) lung volumes is expressed as ml/kg bodyweight at 40 cm H_2O pressure. Data for both Sat PC pool size and lung volumes are shown as *dot plots* with median values. Exposure to the anti-CD18 antibody did not change IA IL-1α–induced improvements in BALF, surfactant Sat PC, or lung volumes (4–6 animals/group, $^{\dagger}p < 0.05$ vs. controls).

that was intermediate between the control lungs and endotoxin-exposed group. There was some variability of Sat PC pool size and lung volumes within the antibody-exposed group; lambs with the least lung inflammation had the lowest Sat PC pools and lung volumes. These results demonstrate that endotoxin-induced inflammatory cell influx is required for mediating inflammation-induced increases in surfactant lipid pool size—an important aspect of the lung maturation phenotype.

Previous experiments from our laboratory demonstrated that endotoxin-induced fetal lung maturation requires direct contact of endotoxin with the lung epithelium (30). After IA injection, endotoxin presumably comes in contact with the fetal respiratory epithelium due to fetal breathing movements that mix amniotic fluid with fetal lung fluid. Fetal lung maturation could result from direct endotoxin signaling of the distal respiratory epithelium via activation of toll-like receptors. Alternatively, endotoxin-induced lung maturation could be a result of signals from activated inflammatory cells recruited to the fetal lung. The results from the present study demonstrate that endotoxin-induced lung maturation requires signals from the recruited inflammatory cells. These recruited inflammatory cells are activated as demonstrated by increased iNOS immunoreactivity and expression of proinflammatory cytokines (31). The predominant inflammatory cell types in fetal lamb lungs after IA endotoxin exposure are neutrophils and the monocytes (7). Preterm fetal lambs at 125-d gestation do not have the alveolar macrophages that are abundant in mature lungs (32). The contributions and mechanisms of lung maturation induced by activated neutrophils and monocytes recruited to the fetal lung await further studies.

The mechanisms of influx of inflammatory cells in the adult lung are complex and involve CD18-dependent and -independent mechanisms dictated by a number of variables, including the nature of the inflammatory stimulus (33). The pulmonary inflammatory cell influx includes pulmonary sequestration of leukocytes and transendothelial and transepithelial migration. The mechanisms of inflammatory cell influx in the preterm fetal lung with its unique pulmonary circulation and innate immune characteristics are unknown. In this study, anti-CD18 antibody significantly decreased neutrophil and monocyte influx in the preterm fetal lung in response to *E. coli* endotoxin but had no effect on inflammatory cell recruitment by recombinant sheep IL-1. This result also suggests that the inhibition of inflammatory cell influx in animals exposed to endotoxin and the anti-CD18 antibody is not due to nonspecific IgG effects. In addition, animals exposed to the anti-CD18 antibody alone had no inflammation or changes in surfactant lipids or proteins. The anti-CD18 antibody did not have systemic effects because the leukocytosis induced by endotoxin or IL-1 was not altered by anti-CD18 antibody. The specificity of the antibody for CD18 was established using human cells (*see* METHODS in the online supplement for details). However, nonspecific interactions with other ligands in the sheep cannot be completely excluded. Because CD18 hetero-dimerizes with other integrins, such as CD11a, CD11b, and CD11c, the relative contribution of these integrins in fetal lung inflammation and maturation cannot be established. Both the transendothelial and transepithelial migration of leukocytes in response to endotoxin were significantly inhibited by anti-CD18 antibody in the fetal lung. Unlike mice with CD18 gene knockout, the fetal lambs in this study exposed to anti-CD18 antibody did not have increased circulating white blood cells (34). There was some heterogeneity in the response to the anti-CD18 antibody, because one of four lambs in each of the 2- and 7-d antibody-treated endotoxin–exposed group had appreciable levels of inflammatory cells in the BALF, whereas the other animals in the groups had essentially no inflammatory cells in BALF. Interestingly, compared with the other antibody-treated lambs in the 7-d endotoxin group, the lamb with increased inflammatory cell had the highest surfactant Sat PC pool size and lung volume. Our data demonstrate that *E. coli* endotoxin– but not IL-1–induced fetal lung neutrophil and monocyte influx is mediated via a CD18-dependent pathway.

In summary, anti-CD18 antibody treatment of preterm fetal lambs decreased IA endotoxin–induced lung recruitment and activation of neutrophils/monocytes, resulting in decreased induction of surfactant lipid pool size.

Conflict of Interest Statement: S.G.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.J.M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.L.J. is employed by ICOS Corporation. ICOS Corporation did not sponsor the study but provided an antibody used in this study under a material transfer agreement. J.P.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.H.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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